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14. ABSTRACT In breast cancer stem-like cells could contribute not only to the initiation of the cancer but also to recurrence because of the resistance of stem cells to chemo/radiation therapy. From several breast cancer cell lines, we have demonstrated the existence of breast cancer stem-like cell subpopulations based on the recognized cell surface markers CD44/CD24 and ABCG2-mediated Hoechst efflux. After radiation we found that a CD44+/CD24- or low subpopulation showed increased clonogenic survival in MCF-7 and HCC1937 cell lines but not in the MDA-MB231 cell line. However, examination of NHEJ activity and expressed proteins of NHEJ did not show any significant difference among the subpopulations suggesting that the increased radiation resistance might not be related to the NHEJ. Furthermore, activation of ATM/ATR pathway was significantly different among the subpopulations of MCF-7, HCC1937, and MDA-MB-231 cell lines and these differences in the activation of ATM/ATR pathway may explain the differential radiation resistance of subpopulations.					
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Introduction

The existence of cancer stem cells is a hypothesis put forth both to explain the initiation of cancer and the recurrence of cancer after treatment. Evidence supporting the presence of cancer stem cells has been developed both in hematologic malignancies and solid tumors. In breast cancer, CD44⁺/CD24^{-low} tumorigenic stem cells have been identified with as few as 100 cells being able to form tumors in mice [1, 2]. These cells exhibit unlimited propagation and could give rise to subpopulations of tumorigenic and non-tumorigenic cells. These CD44 positive cells expressed many known stem cell markers and presence of CD44⁺/CD24^{-low} cells correlated with the survival of breast cancer patients [3]. In addition to breast cancer tissue, cancer stem cell-like cells were also found in carcinoma-derived cell lines[4, 5] . The establishment of various breast cell lines has already indicated that the cells had a clonegenic ability and in some circumstances possessed the characteristics of cancer stem cells. For example, several breast cancer cell lines contain a subpopulation of CD44⁺ /CD24^{-low- positive} with epithelial specific antigens or a side population with stem cell characteristics such as Hoechst efflux and sphere formation. These cells are capable of self-renewal and have the ability to give rise to differentiated cell populations. However, the responses of the breast cancer stem-like cells to chemotherapy and radiotherapy have not been systematically investigated. The understanding of the response characteristics of this type of cell to chemotherapy and radiotherapy could be essential for treatment and cure of breast cancer. In this project, our attempt is to isolate cancer stem cell-like cell populations from cultured breast cancer cell lines to identify the differential molecular responses to radiation and explore new molecular targets for improvement of the radiation therapy. Recently, two publications have addressed the response of breast cancer stem-like cells based on human breast cancer cells line and mouse mammary cells suggesting the resistance of breast cancer stem-like cell and mammary progenitor cells to radiation [6, 7].

Body

I. Characterization of subpopulations in established breast cancer cell lines.

In order to demonstrate the existence of subpopulations in breast cancer cell lines, we examined several breast cancer cell lines with the Hoechst 33342 efflux/ABCG2 and CD44/CD24 surface marker analysis. We first observed side populations by use of Hoechst 33342 efflux in six breast cancer cell lines: a side population, with the percentage of cells in parenthesis, with dye efflux was found in MCF-7 (1.3%), MDA-MB-436 (0.6%), MDA-MB-231 (0.4%), and HCC1937 (0.1%). MDA-MB-453 and HCC38 showed no side population (Figure1). It has been mentioned the Hoechst 33342 efflux is predominately controlled by ABCG2 transporters in mammalian cell membrane. To verify the relationship between ABCG2 and Hoechst 33342 efflux in breast cancer cell lines, we next examined the expression of ABCG2 in cell lines with positive Hoechst 33342 efflux. Figure 2 showed that a small portion of ABCG2 positive cells could be found in some of the cell lines with positive Hoechst efflux, such as MCF-7 (0.7%) and HCC1937 (0.4%) while MDA-MB-231 cells, though exhibiting a side population of positive Hoechst efflux, had no ABCG2 positive cells, suggesting that ABCG2 responsible Hoechst 33342 efflux may be cell line specific.. In addition to Hoechst 33342/ABCG2 analysis, we conducted the analysis of breast cancer cell lines by CD44 and CD24 expression which are well recognized markers for breast cancer stem-like cells. Depending on specific cell lines, the portion of subpopulation with CD44⁺/CD24⁻ ranged from 0 to 97%. (Figure 3A) Two mesenchyme-like breast cancer cell lines MDA-MB-436 and MDA-MB-231 showed highest portion of a CD44⁺/CD24⁻ subpopulation, 97% and 55.3% respectively. For other epithelium-like breast cancer cell lines, the CD44⁺/CD24⁻ subpopulation varied from 0 to 5.9 %. One non-tumorigenic epithelial cell line MCF10A also showed 3.1 % of cells were CD44⁺/CD24⁻ positive. Hence, it seems that mesenchyme-like

breast cancer cell lines contain higher percentages of CD44⁺/CD24⁻ cells than epithelial cancer cell lines and that the CD44⁺/CD24⁻ subpopulation may be present in both tumorigenic and non-malignant breast cells. Further analysis of epithelial specific antigen (ESA) to identify the origin of the cell lines found that in MCF-7 and HCC1937 cells 98% and 93% of cells were ESA positive while in MDA-MB231 cells, only 65% cells were ESA positive indicating that epithelium to mesenchyme transition could accompany the increased portion of CD44⁺/CD24⁻ cells (Figure 3B) .

Does the subpopulation of cells always maintain its CD44⁺/CD24⁻ surface markers or do the surface marker vary/switch with time? To answer this question we analyzed the CD44/CD24 surface marker in presorted MCF-7, MDA-MB-231, and HCC1937 cells. After sorting into CD44⁺/CD24^{-or low} , CD44⁺/CD24⁺ (MDA-MB-231), CD44⁻/CD24^{high} (MCF-7 and HCC1937) populations, cells were cultured for 6 days or two weeks and were then reanalyzed for expression of CD44/CD24 status. Interestingly, 70 to 80% cells maintained the original CD44/CD24 phenotype for 6 days after sorting. However, after two weeks of culture the following transitions were observed: CD44⁺/CD24^{-or low} could change to CD44⁻/CD24^{high} (MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231) and CD44⁻/CD24^{high} (MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231) cells could develop to CD44⁺/CD24^{-or low} cells (Figure 4) . In fact, altered expression of the surface markers CD44/CD24 occurred even in same breast cell line at different passages. For example, with the MCF-7 cell line the percentage of CD44 positive cells increased with increasing cell passage. In HCC1937 cell line, an obvious subpopulation of CD44⁺/CD24⁻ could be found after 15-17 passages with the first culture after purchased from ATCC as passage 1. At passage 21, 98% of cells had become CD44⁺/CD24⁻ (Figure 5, top panels) . This altered expression of surface markers was not limited to CD44 and CD24. The expression of ABCG2 also changed with cell passage number (Figure 5, bottom

panel). Interestingly, the percentage of ABCG2 positive cells decreased in high passages of HCC1937 even though 98% cells were of the CD44⁺/CD24⁻ phenotype. The observation suggests that there may not be a close relationship between the CD44⁺/CD24⁻ or ^{low} phenotype and ABCG2 positive /Hoechst efflux phenotype.

2. Post-radiation survival analysis of CD44⁺/CD24^{-or low} (MCF-7, HCC1937, and MDA-MB231) vs CD44⁻/CD24^{high}(MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231)

According to the original plan, we first attempted to sort the side population by Hoechst 33342 efflux and then further identify these cells by the expression of CD44 and CD24. Detection of Hoechst 33342 requires an ultraviolet laser and unfortunately neither of the two sorters available in our facility, the FACSVantage and the FACS Aria SE, are currently equipped ultraviolet laser. Fortunately, the appropriate lasers have been installed so that it is now feasible to sort by Hoechst 33342 efflux. As alternative, we sorted breast cancer cell lines directly based on the presence of the surface markers CD44 and CD24 which are currently well recognized as markers of stem-like or tumor initiating cells in breast cancer cells. Based on the plating efficiency, the percentage of CD44⁺/CD24^{-or low} cells, and the universality of use of the cell line, we selected three breast cancer cell lines, MCF-7, MDA-MB-231, and HCC1937 (passage 6-17) to sort by their surface marker expression of CD44 and CD24. Sorted cells were cultured for 3 or 5 days and then radiated. Clonogenic survival analysis of the sorted cells is shown in Figure 6. In MCF-7 cells increased radiation resistance was seen in the CD44⁺/CD24^{low} subpopulation compared to CD44⁻/CD24^{high} and non-sorted cells populations at all radiation doses (Figure 6, top panel) . Further analysis of the survival fraction at 2Gy, which approximates clinical doses, showed that the survival fraction in CD44⁺/CD24^{low} subpopulation of MCF-7 and HCC1937 were increased by over 50 % compared to the CD44⁻/CD24^{high} subpopulation. (Figure 6, bottom left and middle panels). In contrast to MCF-7 and HCC1937, higher radiation resistance

was seen in the CD44⁺/CD24⁺ vs. CD44⁺/CD24⁻ subpopulation of MDA-MB231 cells (Figure 6, bottom right panel). Hence, in MDA-MB231 cells more experimental evidence is needed to determine the role of the CD44 and/or CD24 markers in selecting stem-like cells that are radiation resistant. .

To identify further the relationship CD44⁺/CD24^{-or low} phenotype and radiation resistance, we conducted clonogenic survival analysis with MCF-7 cells in culture for 2 weeks after sorting to see if altered CD44 and CD24 phenotype which occurs after longer culture after sorting affects the radiation resistance of cells. The expression of CD44 and CD24 in the CD44⁺/CD24^{-or low} and CD44⁺/CD24⁺ subpopulation before and after culture is shown in Figure 4., After sorting the cells received 2 Gy radiation and clonogenic survival fraction is shown in Figure 7. There was no significant difference between presorted populations (p>0.15, Student T-test) suggesting that the CD44⁺/CD24^{-or low} phenotype is determinant for radiation resistance. On the other hand, when we examined the CD44/CD24 phenotype of non-sorted MCF-7, HCC1937, and MDA-MB-231 cells 10 days after radiation, there was no increase of a CD44⁺/CD24^{-or low} subpopulation.

3. Examine the stem cell marker expression in CD44⁺/CD24^{-or low} vs. CD44⁻/CD24^{high}(MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231)

Does the CD44⁺/CD24^{-or low} phenotype in breast cancer cells represent true stem-like cells? To address this issue, we preliminarily examined the expression of genes related to myeloid stem cells and embryonic stem cells. In MCF-7 cells Notch 1 expression in the CD44⁺/CD24^{low} subpopulation was about 30% higher than in the CD44⁻/CD24^{high} subpopulations (Figure 8). Similar analyses are being undertaken for other genes with increased expression in myeloid and embryonic stem cells.

4. Analysis of end-joining of double-strand DNA breaks in CD44⁺/CD24^{-or low} vs. CD44⁻/CD24^{high} (MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231) subpopulations.

In order to identify differential expressed proteins in the non-homologous end joining (NHEJ) complex in stem-like breast cancer cells, NHEJ activity was first examined in vivo in CD44⁺/CD24^{-or low} and CD44⁻/CD24^{high} (MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231) subpopulations. A plasmid with SV-40 promoter driven luciferase reporter gene, pGL2-Control was linealized by Stu1 to create blunt ends between promoter and luciferase reporter gene. The Stu1 digested plasmid was examined by agarose electrophoresis to verify the linealization. Two days after sorting cells were transfected with linealized reporter plasmid following radiation. The luciferase activity was measured and standardized by expression of co-transfected β -galactosidase. As shown in Figure 9, no increased in vivo NHEJ was seen in cells with in CD44⁺/CD24^{-or low} phenotype. In contrast, increased in vivo NHEJ was found in CD44⁻/CD24^{high} subpopulation of HCC1937 and CD44⁺/CD24⁺ subpopulation of MDA-MB-231 cells. Combined with post-radiation clonogenic survival results, the analysis of in vivo NHEJ suggests that differential clonogenic survival between CD44⁺/CD24^{-or low} sub population and CD44⁻/CD24^{high} subpopulation may not be a simple function of NHEJ, at least, for the acute response phase in MCF-7 and HCC1937 cells after radiation. Only MDA-MB-231 cells showed an increased in vivo NHEJ in the CD44⁺/CD24⁺ subpopulation.

5. DNA damage-repair response after radiation in CD44⁺/CD24^{-or low} vs. CD44⁻/CD24^{high} (MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231) subpopulation of three cell lines.

To investigate the potential differences in NHEJ activity of CD44⁺/CD24^{-or low} vs. CD44⁻/CD24^{high} (MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231) subpopulation, we examined the expression of components of the NHEJ complex by western blot analysis (Figure 10A). The results showed that there

were no significant differences in expression Ku 80, ku70, and PARP-1 between the two subpopulations in the HCC1937 and MDA-MB-231 cell lines. Expression of these components will also be examined in subpopulations of MCF-7 cells. DNA-PK showed a decreased expression by about one fold in CD44⁺/CD24^{-or low} subpopulation in both cell lines after radiation. Examination of expression of DNA ligase IV has been unsuccessful because of the bad quality of antibody available and the investigation of phosphorylation of DNA-PK is in progress.

Because our preliminary data could not completely support the assumption that increased NHEJ is responsible to increased radiation resistance in CD44⁺/CD24^{-or low} subpopulation, we next examined the activation of ATM/ATR pathway which is important for DNA damage/repair responses (Figure 10B). One hour after radiation the phosphorylation of histone 2AX (γ -H2AX) was reduced by about one fold in CD44⁺/CD24^{low} MCF-7 cells compared to CD44⁻/CD24^{high} MCF-7 cells. However, a difference of phosphorylation of H2AX in MCF-7 was not found in MDA-MB231 and HCC1937 cells. (Figure 10B, upper panel). Furthermore, a lack of significant phosphorylation of ATM was found in CD44⁺/CD24^{low} HCC1937 cells (Figure 10B, middle panel). More interesting is that phosphorylation of ATR was much less in CD44⁺/CD24^{-or low} subpopulation of three cell lines than CD44⁻/CD24^{high} cells of MCF-7 and HCC1937 cell lines and CD44⁺/CD24⁺ cells of MDA-MB-231 cell line. These preliminary results suggest that ATM/ATR pathway could contribute to the differential radiation response between CD44⁺/CD24^{-or low} and CD44⁻/CD24^{high} (MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231) subpopulation of the three cell lines. There could also be differential details in the activation of ATM/ATR pathway by radiation among the subpopulations of three cell lines.

Key Research Accomplishments:

In the one year period, we have characterized the cancer-stem cell subpopulation in breast cancer cell lines by cells surface marker and Hoechst efflux and successfully sorted $CD44^+/CD24^{-or\ low}$ (MDA-MB-231, MCF-7, HCC1937) and $CD44^+/CD24^{high}$ (MCF-7 and HCC1937) or $CD44^+/CD24^+$ (MDA-MB-231) subpopulations. We have begun to examine the expression of genes markers representing stem cells in the sorted subpopulations. We have preliminarily examined clonogenic survival of $CD44^+/CD24^{-or\ low}$ (MDA-MB-231, MCF-7, HCC1937) and $CD44^+/CD24^{high}$ (MCF-7 and HCC1937) or $CD44^+/CD24^+$ (MDA-MB-231) subpopulations after radiation. We investigated the NHEJ activity before and after radiation and expression of NHEJ component proteins among the subpopulations. NHEJ does not appear to account for the radiation resistance of stem cell like cells in breast cancer cell lines. We are further exploring the activation of ATM/ATR pathway by radiation among the subpopulation.

Reportable Outcomes

Currently no reportable Outcome.

Conclusions

Breast cancer stem cells exist in several breast cancer cell lines based on recognized surface marker $CD44^+/CD24^{-or\ low}$ or Hoechst efflux/ABCG2. The increased radiation resistance of breast cancer stem-like cells ($CD44^+/CD24^{-or\ low}$) was found in MCF-7 and HCC1937 cell line but not in MDA-MB-231 cells line. The differential radiation resistance among the subpopulation might not rely on the NHEJ activity. Differential activation of ATM/ ATR pathway may contribute to differential radiation resistance among the subpopulations of breast cancer cell lines. Therefore, if NHEJ pathway is further demonstrated not to be responsible for differential radiation resistance among the subpopulations we suggest switching the focus of the study to the radiation-induced activation of the ATM/ATR pathway and identify the ATM/ATR associated proteins after radiation.

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Appendices

Supporting data

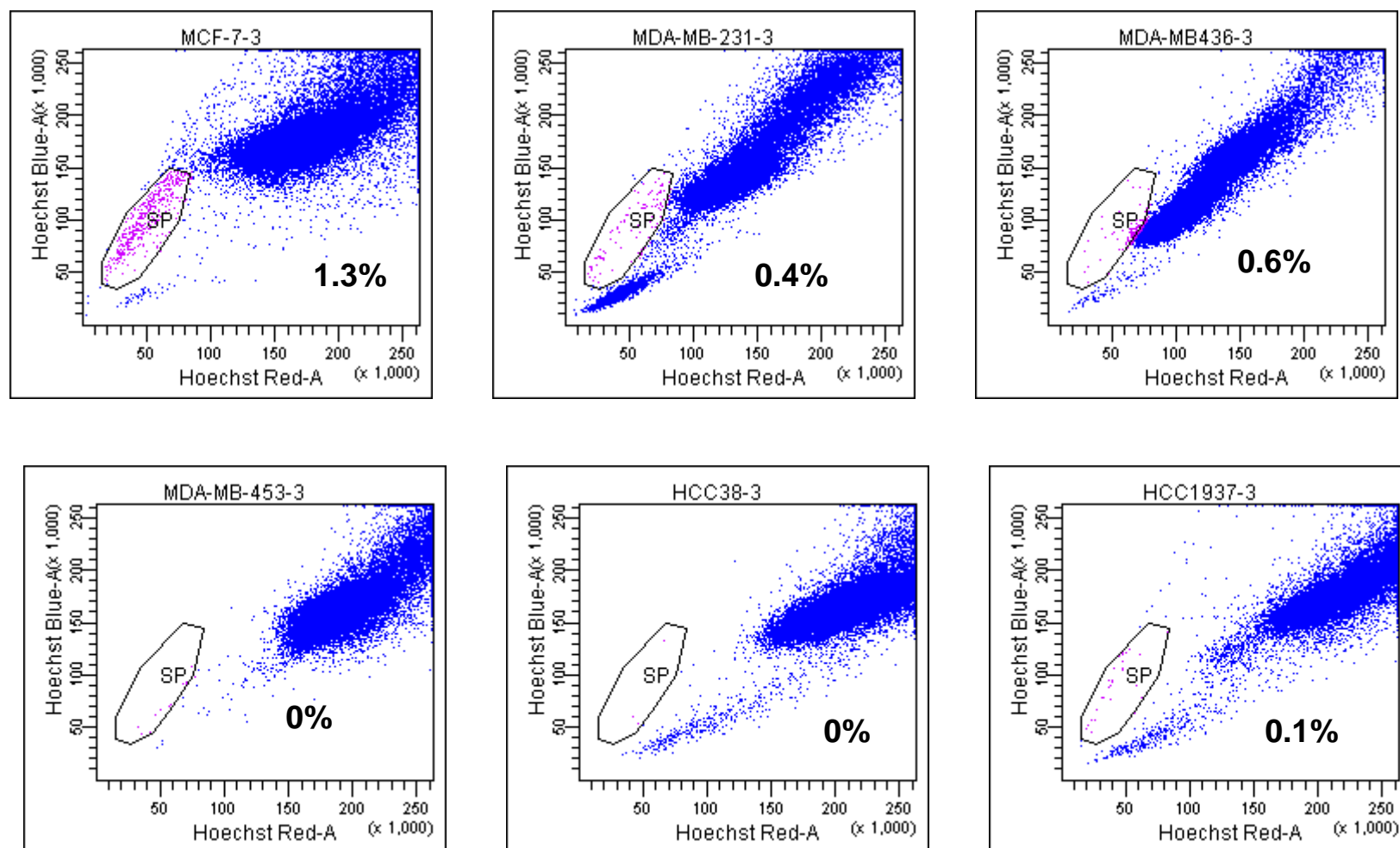


Figure 1. The side populations in breast cancer cell lines. 10^6 cells were loaded with $5\mu\text{g/ml}$ Hoechst 33342 for 90 minutes at 37° . Hoechst efflux was examined by BD LSR-II flow cytometer with UV laser at 360 nm for excitation and 405/20 BP filter for Hoechst blue and 675 LP filter for Hoechst red. The control cells were treated with 50mM Veripamil at the time of staining. The percentage of the side population is listed in each panel.

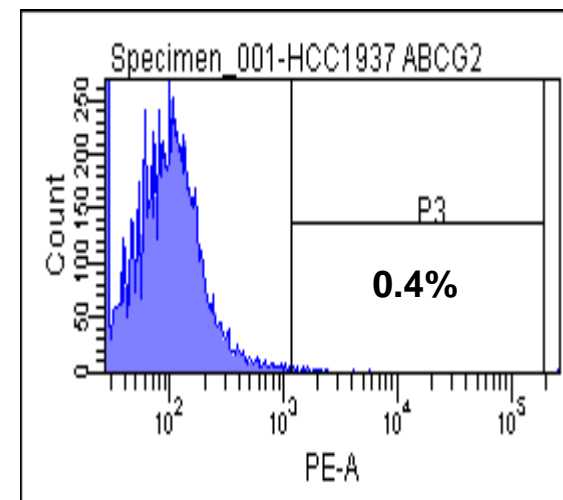
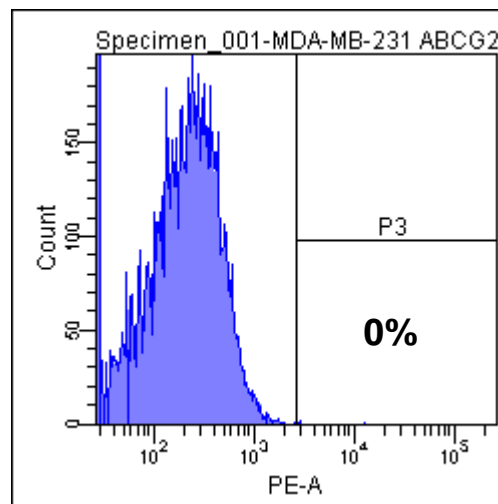
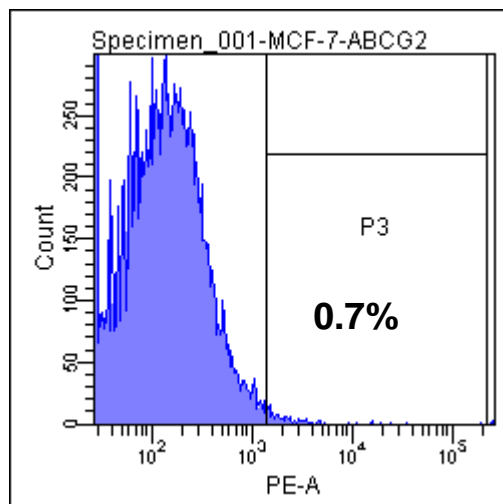
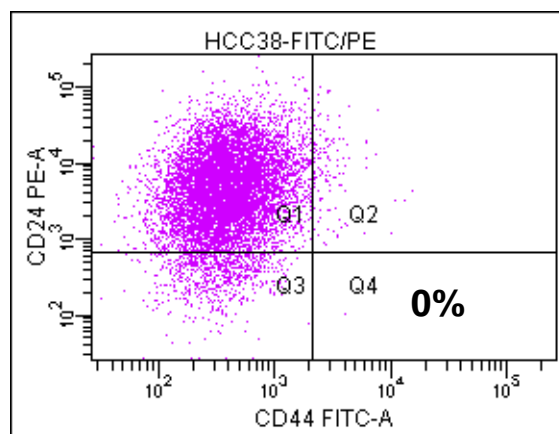
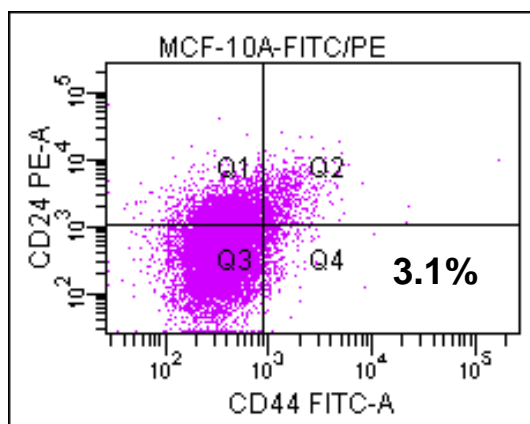
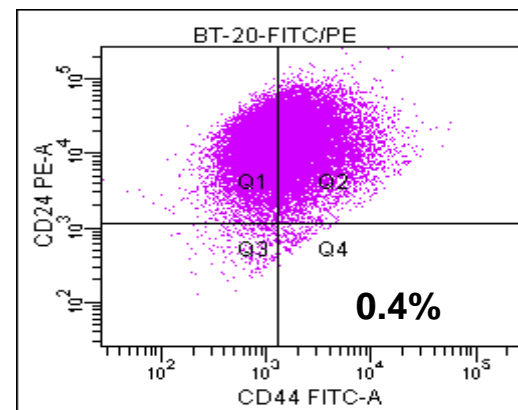
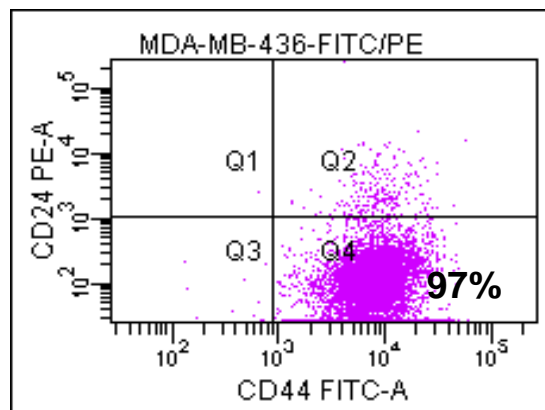
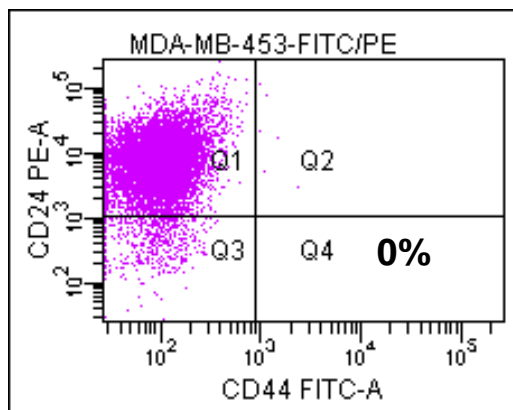
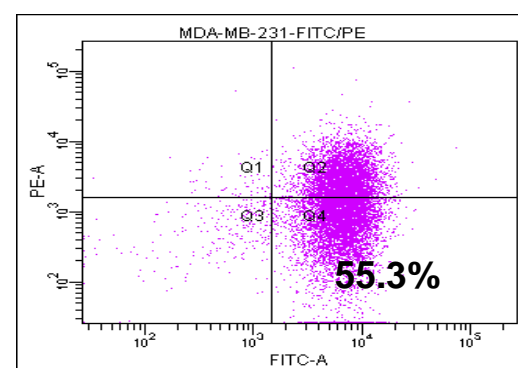
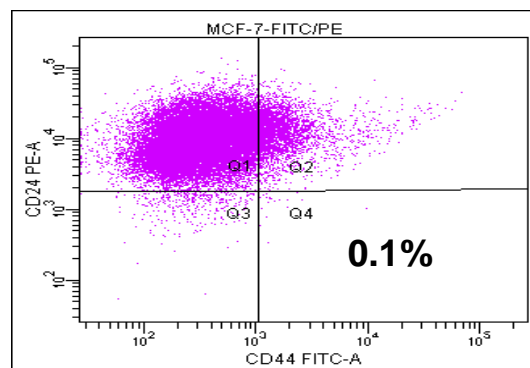
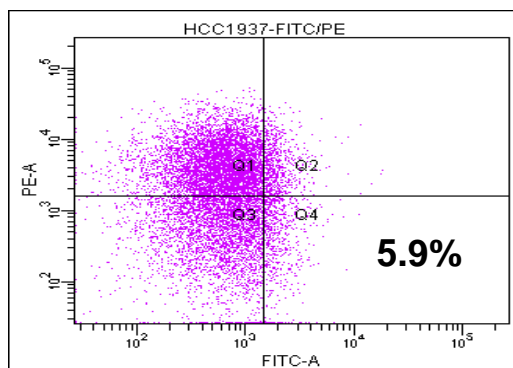


Figure 2 The flow cytometric analysis of ABCG2 expression in MCF-7, MDA-MB-23, and HCC1937 cells. 10^6 cells were stained with PE-conjugated anti-ABCG2 antibody (R&D Systems) according to The manufacturer's recommended protocol. ABCG2 expressed cells were determined against control cells stained with PE-conjugated isotype IgG.

A



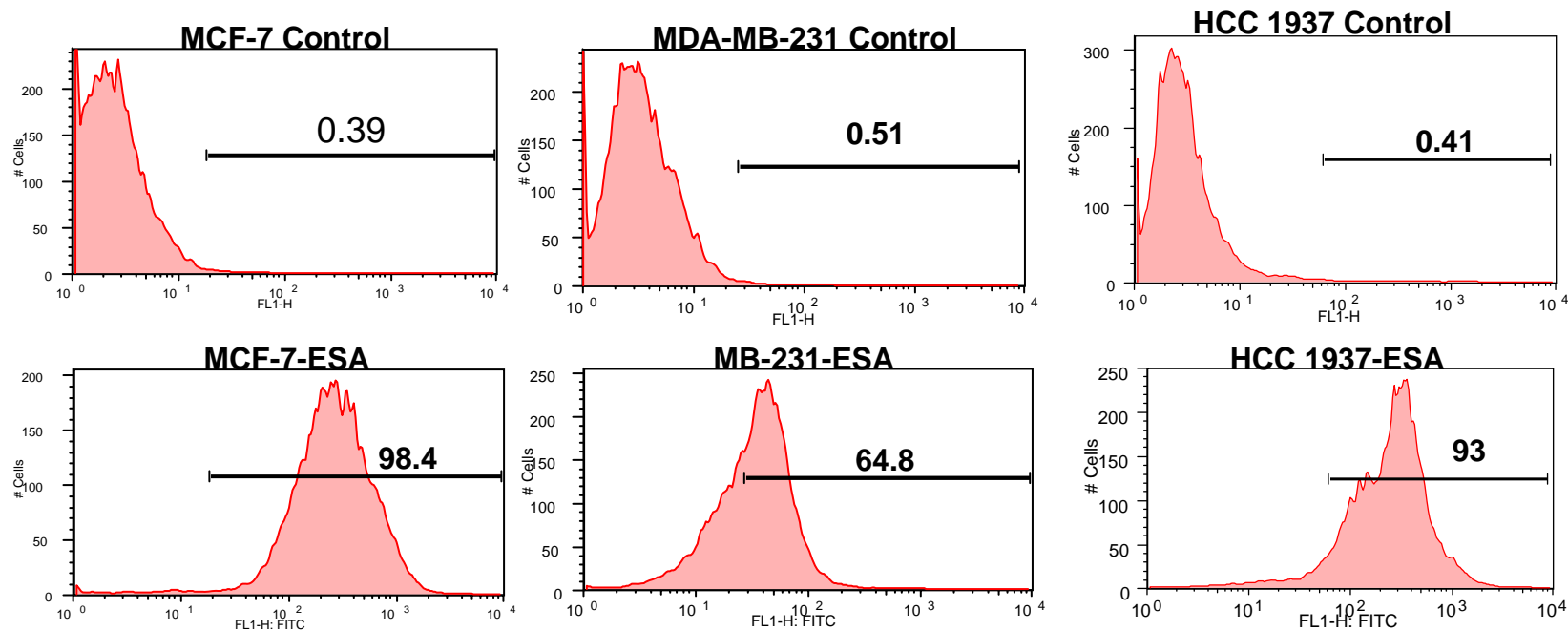
B

Figure 3. Flow cytometric analysis of CD44, CD24, and epithelial specific antigen (ESA)

A. Cells were stained with FITC-conjugated CD44 and PE-conjugated CD24 (BD Biosciences) according to the protocol recommended by the manufacturer. Cells with CD44⁺/CD24⁻ phenotype were determined against cells stained with FITC or PE-conjugated IgG isotypes. Percentage of cells with CD44⁺/CD24⁻ is presented in each panel. **B.** Cells were stained with FITC-conjugated ESA (eBiosciences) and ESA positive cells (bottom panel) were determined against control cells stained with IgG isotype (top panels). The number in each panel represents the percent ESA positive cells.

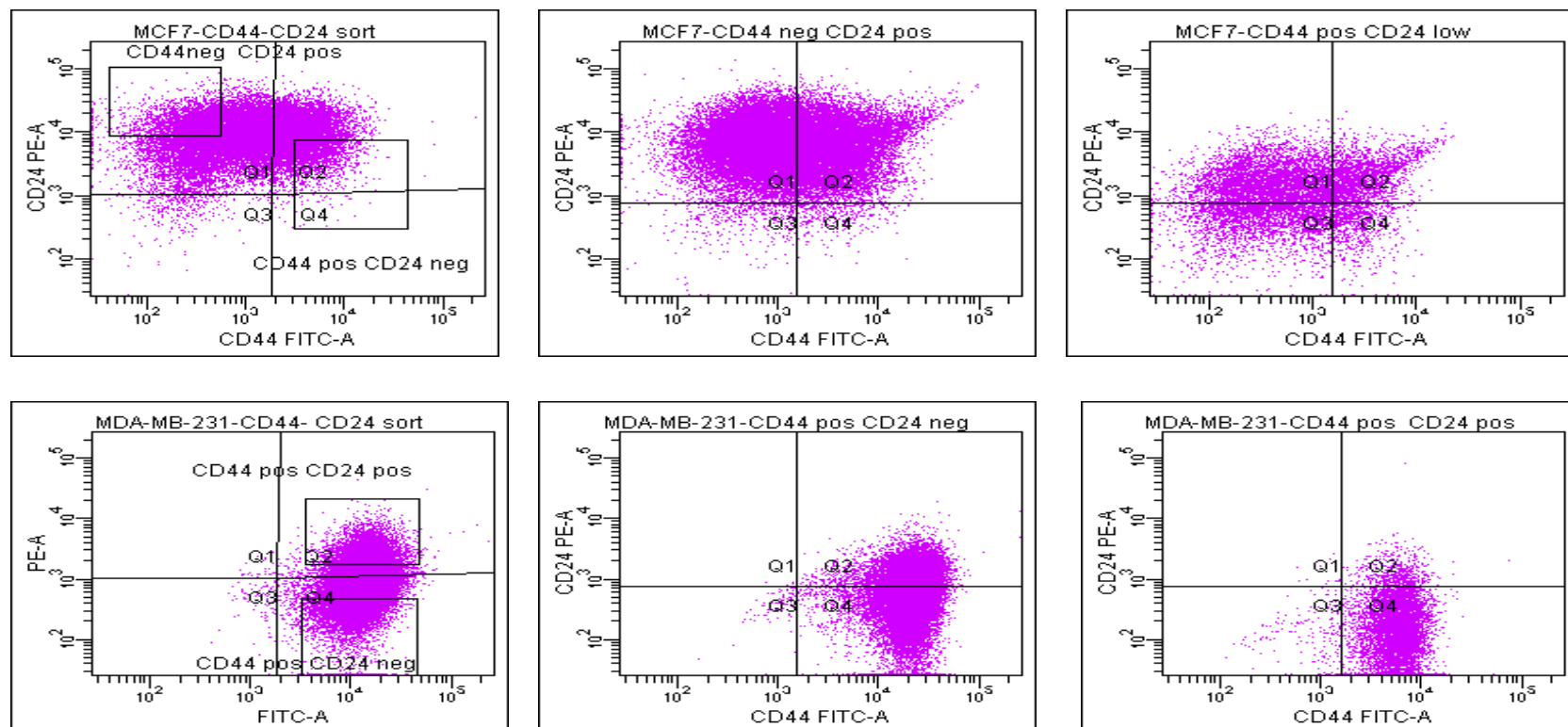


Figure 4. The switch of CD44/CD24 expression in presorted cells.

MCF-7 (Top panel) and MDA-MB-231 (bottom panel) cells were sorted by the surface markers CD44 and CD24 as shown in left column and then were cultured. After 2 weeks the presorted cells were re-examined for expression of CD44 and CD24 with the analysis of the original CD44⁺/CD24^{low} cells in the middle column and the analysis of the original CD44⁺/CD24^{high} cells in the right column.

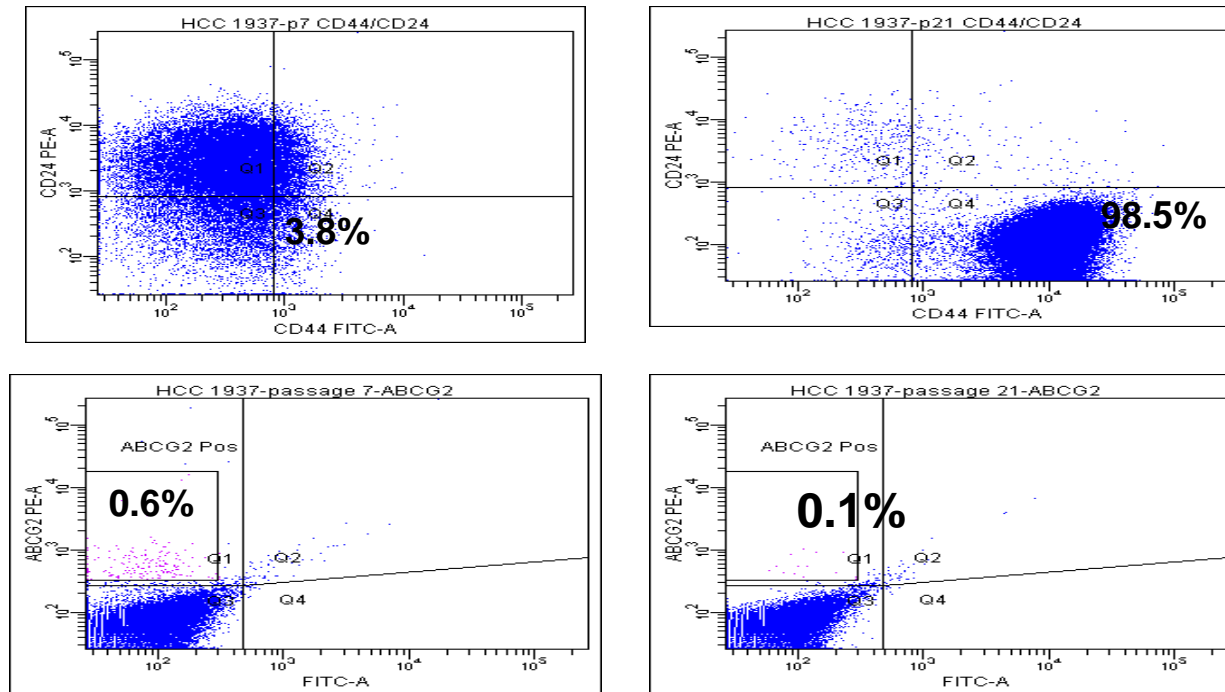


Figure 5. Altered expression of CD44/CD24 and ABCG2 surface markers with early and late passage of the HCC1937 cell line. Early (passage 7) and late (passage 21) HCC1937 cells were stained with CD44/CD24 (top panels) or ABCG2 (bottom panels). The percentage of cells with CD44⁺/CD24⁻ phenotype or positive ABCG2 were determined by flow cytometric analysis.

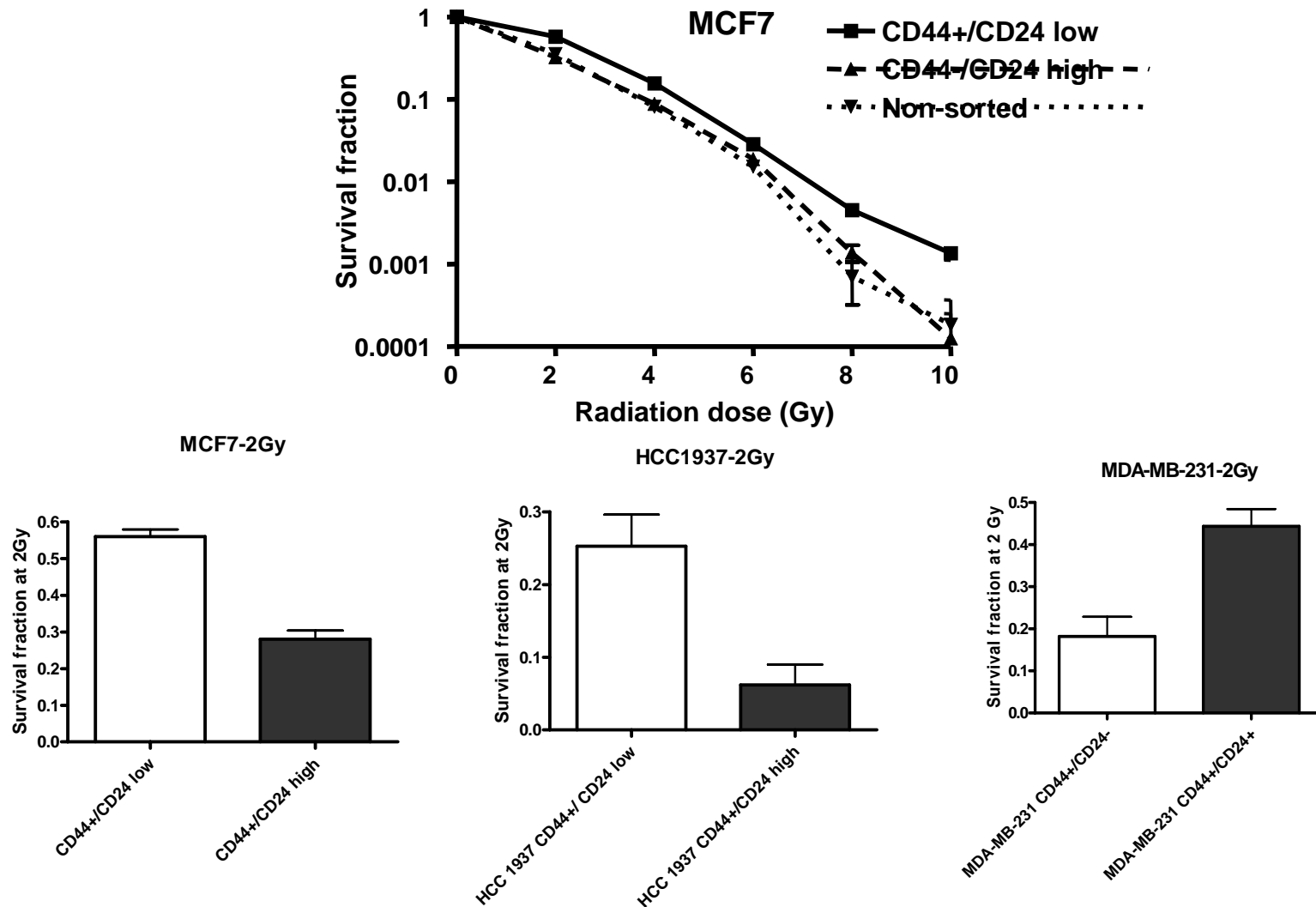


Figure 6. Post-radiation clonogenic survival analysis of CD44⁺/CD24^{low} subpopulation vs. CD44⁻/CD24^{high} (MCF-7 and HCC1937) and CD44⁺/CD24⁺ (MDA-MB-231) subpopulations

Cells were radiated 5 days after sorting and clonogenic survival analysis was conducted. On day 14 after radiation the colonies were counted and survival fraction was calculated as colony formed divided by cell number seeded and corrected for plating efficiency .

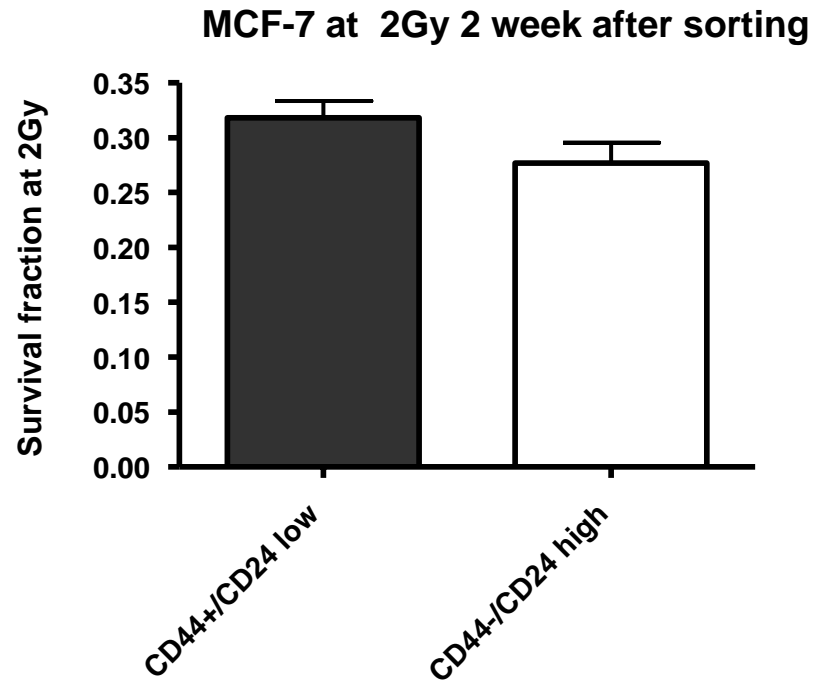


Figure 7 Post-radiation clonogenic survival analysis of MCF-7 subpopulations 2 week after sorting.

MCF-7 cells were sorted by CD44 and CD24 as CD44⁺/CD24^{low} and CD44⁻/CD24^{high} subpopulations. After 2 week of culture, cells were exposed to radiation at 2 Gy. Clonogenic survival analysis was conducted and survival fraction at 2 Gy was calculated as described in Figure 6. Each column represents mean of three independent experiments \pm SD.

Notch -1 expression

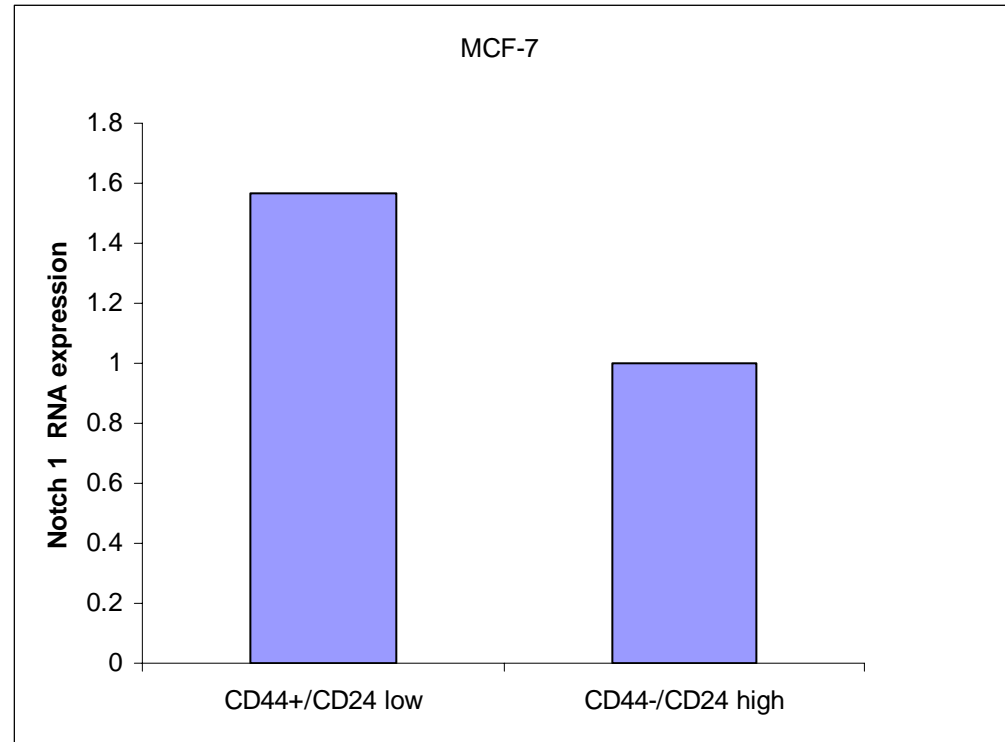


Figure 8. Notch 1 expression in CD44⁺/CD24^{low} and CD44⁻/CD24^{high} subpopulations of MCF-7 cells. MCF-7 cells were sorted according to the expression of CD44 and CD24. After 2 days of culture of the sorted cells RNA was extracted and real time RT-PCR was conducted. The relative expression of Notch1 was calculated by Δ ct of Notch1 over Δ ct of beta actin.

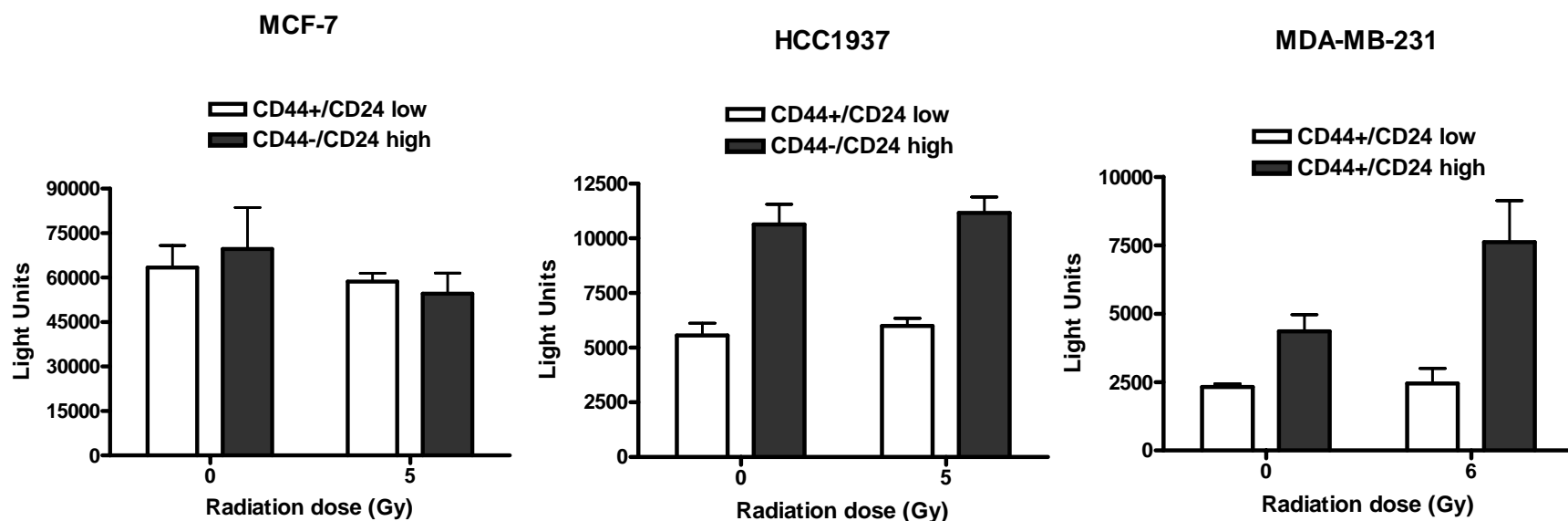


Figure 9. Analysis of In vivo ligation analysis activity among subpopulations of MCF-7, HCC1937, and MDA-MB-231 cell lines.

A plasmid with SV-40 promoter driven luciferase reporter gene, pGL2-Control was

linealized by Stu1 to create blunt ends between promoter and luciferase reporter gene. The linealized plasmid was transfected into subpopulations of MCF-7, HCC193, and MDA-MB-231 cells prior and post radiation. The luciferase activity was measured 36 hours after transfection. The luciferase activity was standardized by β -gal activity for transfection efficiency and represents as light unit.

A

MDA-MB-231

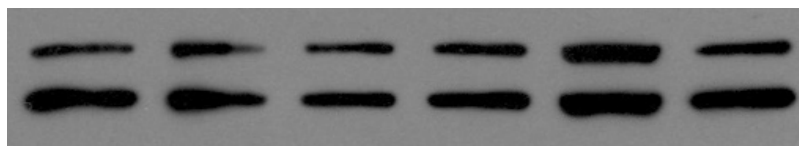
HCC1937

CD44+/	CD44-/	CD44+/	CD44+/	CD44-/	CD44+/	
CD24- or	CD24	CD24- or	CD24- or	CD24	CD24- or	
low	high	low	low	high	low	
0	5	5	0	5	5	Gy

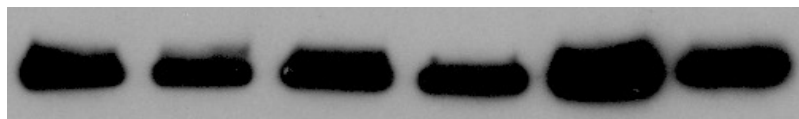
DNA-PK



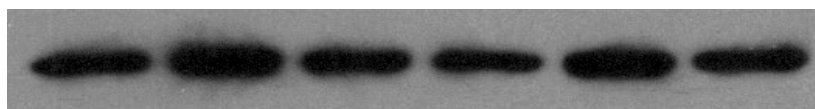
Ku80/70



PARP-1



Actin



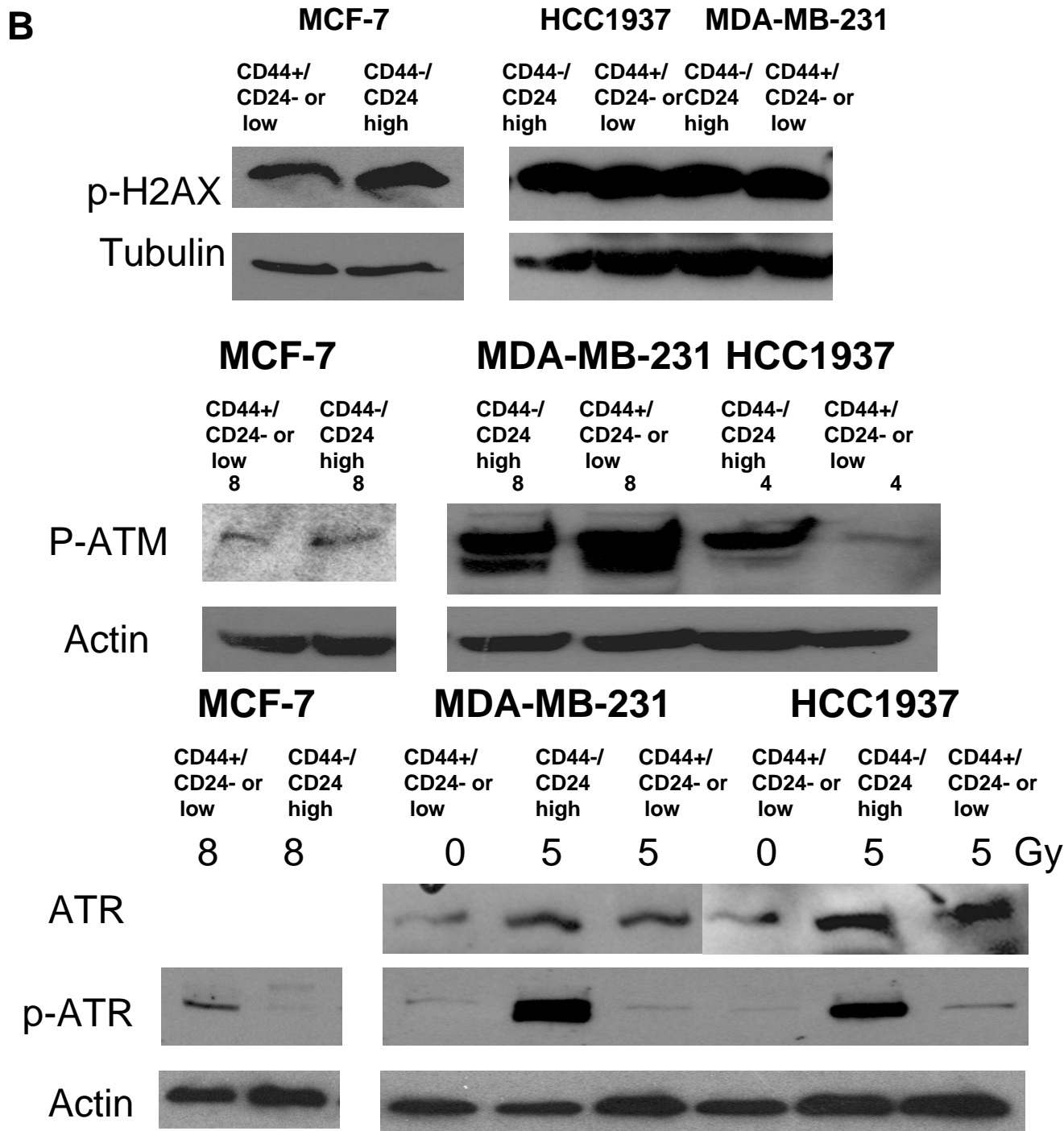


Figure 10. Western blot analysis of components of NHEJ and phosphorylated H2AX, ATM, and ATR. Cells were sorted by FITC-conjugated CD44 and PE-conjugated CD24 and continued in culture for 5 days. The whole cell extract (MCF-7) or nuclear extract (MDA-MB-231 and HCC1937) was prepared one hour after radiation. Western analyses was conducted to detect the expression of components of NHEJ (A) and phosphorylated H2AX, ATM, and ATR (B).

Note: There were not enough MCF-7 cells to isolated nuclear extract after sorting.